Peptide Immunization Indicates that CD8$^+$ T Cells are the Dominant Effector Cells in Trinitrophenyl-Specific Contact Hypersensitivity

Stefan Martin,* Michael B. Lappin,* Jochen Kohler,† Virginie Delattre,* Cornelia Leicht,* Tobias Preckel,† Jan C. Simon,* and Hans Ulrich Weltzien*†

*Clinical Research Group, Department of Dermatology, University of Freiburg, Freiburg, Germany; †Max-Planck Institute for Immunobiology, Freiburg, Germany

The identity of the effector T cell population involved in contact hypersensitivity is still questionable with evidence promoting both CD4$^+$ or CD8$^+$ T cells. Previous experimental studies have relied on the *in vivo* depletion of T cell subsets using antibody, or the use of knock-out mice with deficiencies in either CD4$^+$ or CD8$^+$ T cell-mediated immunity. To address the role of the class I- and class II-mediated pathways of T cell activation in contact hypersensitivity responses in mice with an intact immune system, we utilized various trinitrophenyl-derivatized peptides, which bind specifically with H-2K^b (major histocompatibility complex class I) or H-2I-A^b (major histocompatibility complex class II). The subcutaneous injection of major histocompatibility complex class II-specific, but not of class I-binding, hapten-derivatized peptides in incomplete Freund's adjuvant induced specific, albeit low, contact hypersensitivity responsiveness to trinitrochlorobenzene. When bone-marrow-derived dendritic cells, however, were pulsed with the same peptides and administered intradermally, the opposite result was observed, namely that the class I binding peptides induced contact hypersensitivity responses similar to that observed after epicutaneous trinitrochlorobenzene application. In contrast, dendritic cells pulsed with major histocompatibility complex class II binding peptides did not reproducibly sensitize for contact hypersensitivity responses. Surprisingly, both immunization protocols efficiently induced CD8$^+$ effector T cells. These results support the notion that CD8$^+$ T cells are the dominant effector population mediating contact hypersensitivity responsiveness and that the CD4$^+$ T cell subset only contributes little if at all. *Key words*: allergy/dendritic cells/hapten/rodent. *J Invest Dermatol 115:260–266, 2000*

Cutaneous exposure to reactive haptens induces T cells which initiate contact hypersensitivity (CHS) responses on re-challenge. The depletion of either CD4$^+$ or CD8$^+$ T cells by antibody *in vivo*, and studies in major histocompatibility complex (MHC) class I and class II knockout mice, both support CD8$^+$ T cells as being the main effector population in CHS (Gocinski and Tigelaar, 1990; Bour *et al*, 1995). Thus, hapten-specific CD8$^+$ T cells developed in the absence of CD4 T cell help, in mice depleted of CD4$^+$ T lymphocytes with antibody (Xu *et al*, 1997), and effective CHS sensitization was achieved by the transfer of hapten-derivatized MHC class I$^+$/II$^-$ dendritic cells (DC) into syngeneic recipient mice (Kolesaric *et al*, 1997). Cytokine production is important in the regulatory and effector activity of CD4$^+$ and CD8$^+$ T lymphocytes. T cells induced by skin painting with hapten (Xu *et al*, 1996) or by the transfer of derivatized Langerhans cells (Xu *et al*, 1997), showed divergent cytokine profiles, with CD4$^+$ T cells secreting interleukin (IL)-4 and CD8$^+$ T cells producing interferon (IFN)-γ.

It is impossible, however, to rule out an effector role for CD4$^+$ T cells. Depletion experiments with low concentrations of anti-CD4 antibody (Gocinski and Tigelaar, 1990), eliminated only those CD4$^+$ T cells with high-density CD4 expression. Rather than causing enhanced CHS, this treatment suppressed responses (Gocinski and Tigelaar, 1990). A direct involvement of CD4$^+$ T cells in CHS has been shown by adoptive transfer experiments (Hauser, 1990). In addition, mice lacking the CD4$^+$ gene through targeted disruption, showed reduced CHS responses compared with normal syngeneic controls (Kondo *et al*, 1996), suggesting an important role for CD4$^+$ T cells in the induction of CHS.

The pathway of immune recognition of chemical haptens is well described. Haptens binding directly to peptides in MHC binding grooves provide the principal immunogenic determinants in CHS responses (von Bonin *et al*, 1992; Martin *et al*, 1993; Cavani *et al*, 1995; Kohler *et al*, 1995; Weltzien *et al*, 1996). Trinitrobenzenesulfonic acid (TNBS)-specific CD4$^+$ and CD8$^+$ T cell clones recognize derivatized peptides though the trinitrophenyl (TNP) group, and to a greater or lesser extent, through amino acid determinants on the carrier peptides (Martin *et al*, 1992, 1993; Kohler *et al*, 1995; Weltzien *et al*, 1996).

Chemically synthesized peptides, bearing a reactive TNP group on a single lysine residue, have been used to assess the specificity of

Manuscript received March 22, 1999; revised March 31, 2000; accepted for publication April 25, 2000.

Reprint requests to: Dr. S. Martin, Department of Dermatology, University of Freiburg, Haupstrasse 7, D-79104 Freiburg, Germany. Email: martin@haut.ukl.uni-freiburg.de

Abbreviations: CHS, contact hypersensitivity; CASN, Con-A induced rat spleen supernatant; DC, dendritic cell; TNCB, trinitrochlorobenzene; TNBS, trinitrobenzenesulfonic acid.

0022-202X/00/$15.00 · Copyright © 2000 by The Society for Investigative Dermatology, Inc.

T cell responses to TNP haptens *in vitro* (von Bonin *et al*, 1992, 1993; Martin *et al*, 1992, 1993; Kohler *et al*, 1995; Weltzien *et al*, 1996). In addition, there is evidence that derivatized peptides can sensitize for CHS (Kohler *et al*, 1995; Weltzien *et al*, 1996). The derivatization of peptides that bind specifically to MHC class I or II provides a technique to investigate the T cell subset requirements of CHS. Because of the conflict in the data from earlier studies (Gocinski and Tigelaar, 1990; Bour *et al*, 1995; Kondo *et al*, 1996; Xu *et al*, 1996, 1997; Kolesaric *et al*, 1997), we decided to examine the ability of hapten-derivatized peptides, which bind specifically to MHC class I (H-2K^b) or class II (H-2I-A^b) to sensitize mice for CHS responses *in vivo*. This system has the advantage that the CHS response is induced in mice with an intact immune system.

MATERIALS AND METHODS

Mice Female C57BL/6 mice aged 6–8 wk (Charles River, Sulzfeld, Germany) were housed in the animal facility of the Max Planck Institute, Freiburg. All the experimental procedures carried out were in accordance with the Max-Planck Institute and the University of Freiburg guidelines on animal welfare.

Media and chemicals RPMI 1640 (Gibco, Eggenstein Germany) was supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 25 mM HEPES buffer (Gibco), and 50 μg penicillin–streptomycin per ml (Gibco) (cRPMI). TNBS was from Sigma Immunochemicals (Deisenhofen, Germany) and trinitrochlorobenzene (TNCB) was obtained from the Department of Chemistry, University of Freiburg.

Peptides The synthesis, purification and quality control of TNP-peptides specific for either H-2I-A^b or H-2K^b have been described before (Martin *et al*, 1992, 1993, 1995; Kohler *et al*, 1995, 1997). The following peptides were used in this study with asterisks indicating the TNP-modified Lys residues: (i) I-A^b-binding (Kohler *et al*, 1995, 1997): Nase-TNP (YADGK*MVNEALVR), PCC-TNP (GFSYTDANK*NK-GIT), IgGVH-TNP (GGNADFK*TPATLT), CH-7-TNP (AAAA-FK*TPAAAA); (ii) K^b-binding (Martin *et al*, 1992, 1993, 1995): 04-TNP (SIIK*FEKL), 07-TNP (SIINFEK*L), Y3K4-TNP (GGYK*FGGL) and V4s-TNP (RGYK*YQGL), M4LY3-TNP (SMYK*FGEL).

DC cultures DC were generated following the protocol of Inaba *et al.* (1992), with minor alterations. Bone marrow was harvested from the tibia and femur of C57BL/6 mice (n=3–4). The cells were resuspended at 1×10^6 cells per ml cRPMI-1640 (Gibco) with 40 ng recombinant murine granulocyte-macrophage-colony-stimulating factor per ml (PromoCell, Heidelberg, Germany), and 100 ng recombinant murine IL-4 per ml (PromoCell). Cells were cultured in 1 ml aliquots in 24 well culture plates (Greiner, Frickenhausen, Germany) and fed on days 3 and 5 of culture, by replacing half the medium in each well with fresh cRPMI with granulocyte-macrophage-colony-stimulating factor and IL-4. On day 3, nonadherent cells were aspirated off following gentle swirling of the plate. Loosely adherent cells, including DC, were harvested by gentle pipetting, on day 6 of culture. DC were washed once and resuspended at approximately 5×10^5 cells per ml in cRPMI. Eight milliliter volumes of the cell suspension were underlayed with 2 ml 14.5% Metrizamide (Boehringer Ingelheim, Heidelberg, Germany) in a 14 ml conical bottomed tube (Becton Dickinson, Heidelberg, Germany) and centrifuged at room temperature (22°C) for 20 min at $600\times g$. The low buoyant density cells were collected, washed twice, and resuspended for use. DC preparations were characterized by flow cytometry and consisted typically of >80% CD11c^{hi}, I-A^{bhi}, GR1^{lo}, Mac3^{lo} cells.

Induction of CHS responses Peptides were solubilized in phosphate-buffered saline (PBS) at 2 mg per ml and then emulsified in an equal volume of incomplete Freund's adjuvant (IFA). Mice were injected subcutaneously in the base of the tail with 100 μl of peptide or peptide-free control emulsion. Alternatively, DC-enriched populations were prepared from day 6 or 7 bone marrow cultures using Metrizamide centrifugation. DC were washed twice in cRPMI, resuspended to $1–10\times10^6$ cells per ml in cRPMI. For TNBS derivatization DC were washed twice in serum-free PBS and incubated with 1 mM TNBS at 37°C for 7 min (TNBS-DC). Negative control DC were incubated with an equal volume of PBS (null-DC). For peptide treatment, DC remained in cRPMI and were incubated for 2 h at 37°C with 100 μM of peptides specific for either K^b (04-TNP, 07-TNP, Y3K4-TNP, V4s-TNP) or I-A^b (Nase-TNP, IgGVH-TNP). DC were then washed twice in cRPMI and twice in PBS, resuspended in PBS, and $1–3\times10^5$ cells were injected intradermally into two sites on the shaved abdomen of C57BL/6 mice (n=5) on 2 subsequent days. In some experiments mice (n=5) were painted on the abdomen with 100 μl of either 7% TNCB or 3% oxazolone or vehicle (acetone or ethanol, respectively). Five days after the second DC injection, or 6 d after the injection of peptide in IFA, the ears of all mice were measured with an engineers micrometer (Mitutoyo, Leonberg, Germany) prior to challenge on the dorsum of both ears with 1% TNCB or 1% oxazolone in vehicle. The mean increase in ear thickness ±SEM (n=6–10) was measured 24 and 48 h following challenge. The measurement of CHS responses was completed in a "blind" manner.

IL-2 secretion by TNP specific hybridomas DC were γ-irradiated with 3×10^3 rad, and were either left untreated, pulsed for 2 h with 100 μM peptide (04-TNP or IgGVH-TNP) or were modified with TNBS as described above. Two hybridoma cell lines were used as responders; IA-8, which was CD8$^+$ and specific for K^b-binding peptides modified with TNP in position 4 (von Bonin *et al*, 1993), and IT/H6-A11, which was CD4$^+$ and specific for IgGVH-TNP in an I-A^b restricted context (Kohler *et al*, 1997). After extensive washing 5×10^5 DC stimulators were mixed with 1×10^5 hybridoma cells in 96-well round bottomed plates and incubated at 37°C. After 24 h 100 μl supernatant was transferred to a fresh 96-well round-bottomed plate and frozen at –20°C. After thawing, 5×10^3 CTLL-cells/well were added (Grabstein *et al*, 1986; von Bonin *et al*, 1993). CTLL cells cultured in the presence of 10, 2, 0.4, or 0.08% concavalin A-induced rat spleen supernatant (CASN) served as controls. After 24 h of incubation, the cultures were pulsed with 0.5 μCi of ^{3}H-thymidine (TdR) for 14 h. The cells were then harvested onto filters and the incorporation of ^{3}H-TdR was determined using a beta counter (Topcount; Canberra Packard, Dreieich, Germany). The means of triplicate cultures were calculated, standard deviation (SD) ≤12%.

TNP-specific fluorescence-activated cell sorter staining DC were either left untreated or pulsed with 100 μM of TNP-modified peptides (04-TNP, 07-TNP or IgGVH-TNP) for 2 h at 37°C. TNBS-modified cells were prepared as described above. After extensive washing the cells were stained in 96-well round-bottomed plates at 1×10^5 cells per well with a 1:30 dilution of a rabbit anti-TNP serum (Preckel *et al*, 1997) and subsequently with fluorescein isothiocyanate-labeled donkey anti-rabbit Ig (Dianova, Hamburg, Germany). DC were counterstained with phycoerythrin-labeled CD11c (Pharmingen). Following a final wash the cells were analyzed with a FACScan, applying Cell Quest software (Becton Dickinson). Dead cells were gated out by FSC/SCC parameter and 10^4 cells analyzed.

Analysis of effector cell populations Mice were immunized with untreated or peptide-pulsed DC as described above. On day 5 spleen and axillary/maxillary/auricular lymph nodes were removed, pooled and single cell suspensions prepared. Total spleen or lymph node cells (4×10^5 per well) were restimulated in 96-well U-bottom plates (Greiner, Nürtingen, Germany) with irradiated (3×10^3 rad), TNBS-modified syngeneic spleen cells (3×10^5 per well). Three days later, cells were harvested and a 4 h chromium release assay was performed as previously described (Martin *et al*, 1992). Briefly, $1–2\times10^6$ target cells (EL4 lymphoma) were labeled for 90 min at 37°C with 50 μCi $Na_2\,^{51}CrO_4$ in the presence or absence of 5 μg synthetic TNP peptide. After repeated washes, 2×10^3 EL-4 cells were incubated with 6×10^4 total spleen or lymph node cells in 96 well U-bottom plates for 4 h. Supernatants were harvested and chromium release determined in a γ-counter (Topcount).

For the analysis of cytokine production different lymphoid organs were removed on day 5 after s.c. injection of TNP-peptides in IFA or intradermal injection of TNP-peptide pulsed DC. Single cell suspensions were prepared and CD4$^+$ or CD8$^+$ T cells removed by magnetic bead depletion according to the manufacturer's protocol (Dynal, Hamburg, Germany). Depletion efficiency was typically 95%. ELISA plates (Greiner) were coated with anti-IFN-γ or anti-IL-4 capture antibodies (Pharmingen) at 4°C overnight. Plates were then washed 4× with PBS/0.05% Tween-20 and blocked with PBS/10% fetal bovine serum at room temperature for 30 min. Plates were washed 3× with PBS/Tween. Recombinant cytokine standards (Pharmingen) and samples (culture supernatant) were diluted and incubated at room temperature for 2–4 h. Plates were washed 4× with PBS/Tween and the biotinylated detection antibodies added. After 1 h incubation at room temperature plates were washed and streptavidin-coupled horseradish peroxidase (Dianova, Hamburg) was added for 1 h at room temperature. Plates were washed 4× with PBS/Tween and substrate (3,3′,5,5′ TBD (Sigma) in phosphate citrate buffer with H_2O_2) was added.

Table I. MHC class II but not class I binding peptides sensitize for contact sensitivity to TNCB when injected in IFA[a]

	Sensitization	Peptide binding specificity	Challenge	Ear swelling (mm × 10^{-2})
Experiment 1	IFA		TNCB	1.75 ± 1.3
	TNCB		TNCB	**25.5** ± 1.5
	Nase-TNP	I-A^b	TNCB	**7.8** ± 0.6
	PCC-TNP	I-A^b	TNCB	**8.0** ± 2.0
	IgGVH-TNP	I-A^b	TNCB	5.5 ± 0.7[b]
	CH-7-TNP	I-A^b	TNCB	**6.0** ± 0.7
Experiment 2	IFA		TNCB	3.8 ± 0.6
	IFA		Ox	0.3 ± 2.0
	Nase	I-A^b	TNCB	3.0 ± 0.8
	Nase-TNP	I-A^b	TNCB	**7.3** ± 0.8
	Nase-TNP	I-A^b	Ox	0.8 ± 1.3
	Ox		Ox	**23.7** ± 1.6
	TNCB		TNCB	**18.8** ± 2.7
	Ox		TNCB	2.7 ± 0.5
	Y3K4-TNP	K^b	TNCB	3.5 ± 0.4
	V4s-TNP	K^b	TNCB	4.3 ± 0.3
	M4LY3-TNP	K^b	TNCB	3.3 ± 0.5

[a]All peptides used have been previously described (see *Materials and Methods*). Peptides were dissolved in PBS (2 mg per ml) and were emulsified in equal volumes of IFA. Peptide or peptide free control emulsions (100 µl) were injected s.c. in the base of the tail 6 d prior to challenge with 20 µl of 1% TNCB on the dorsum of both ears. Data represent mean ear swelling responses ±SD (n=3) 24 h after challenge. In bold are values greater than control + 3 standard deviations.

[b]In two other experiments, IgGVH resulted in responses comparable with Nase-TNP or PCC-TNP.

The assay was developed for 10 min in the dark and the reaction stopped by addition of 0.5 M H_2SO_4. OD at 405 nm was measured in a microplate reader (Dynatech MR 5000; Dynatech Technologies, Denkendorf, Germany) and cytokine concentrations calculated using the Biolinx software (Dynex Technologies).

RESULTS

Class II but not class I binding peptides mediate induction of CHS following subcutaneous injection in IFA Studies in lymphocyte subset depleted mice have demonstrated the importance of IFN-γ secreting $CD8^+$ T lymphocytes in CHS responses (Gocinski and Tigelaar, 1990; Bour *et al*, 1995; Xu *et al*, 1996, 1997; Kolesaric *et al*, 1997), and suggest a regulatory role for the $CD4^+$ T lymphocyte population. Data from studies in CD4-depleted mice (Gocinski and Tigelaar, 1990), CD4-deficient mice (Kondo *et al*, 1996), and from studies using TNP-modified-MHC class II binding peptides (Kohler *et al*, 1995), however, suggested an effector role for $CD4^+$ T cells in CHS responses. To clarify this apparent conflict, we examined the efficacy of TNP-modified peptides with binding specificity for either K^b or I-A^b to sensitize for CHS to TNCB. As demonstrated (**Table I**), the subcutaneous (s.c.) injection of the I-A^b binding peptides Nase-TNP, PCC-TNP, IgGVH-TNP, or CH-7-TNP, sensitized mice for CHS to TNCB (**Table I**, experiment 1). Not surprisingly, peptide priming was less efficient than skin painting with TNCB, but these weak responses were reproduced in three independent experiments. In contrast, in **Table I**, experiment 2, H-2K^b binding TNP peptides, such as Y3K4-TNP, V4s-TNP or M4LY3-TNP reproducibly failed to transfer ear-swelling responses when given s.c. in IFA. In the same experiment, the MHC class II-binding peptide Nase-TNP again resulted in sensitization for TNCB. Control experiments involving oxazolone as an independent hapten or the unmodified Nase peptide revealed the hapten-specificity of sensitization.

Class I binding peptides induce CHS when presented on professional antigen-presenting cells (APC) To investigate the discrepancy between our finding, that upon s.c. injection class II but not class I binding peptides could mediate CHS *in vivo*, and published data (Gocinski and Tigelaar, 1990; Bour *et al*, 1995; Xu *et al*, 1996, 1997; Kolesaric *et al*, 1997), suggesting the importance of class I-mediated pathways in CHS responses, we used a more defined system in which BM-derived syngeneic DC were pulsed with a variety of MHC class I and II binding TNP-modified peptides, and then injected intradermally into syngeneic recipients. Although the s.c. injection of the MHC class I-binding peptides Y3K4-TNP or V4s-TNP in IFA failed to mediate CHS, DC pulsed with the same or other MHC class I-binding peptides (04-TNP, 07-TNP) induced strong TNP-specific responses similar to that observed after epicutaneous sensitization with TNCB (**Fig 1**). In contrast, the injection of DC pulsed with the MHC class II-binding peptide IgGVH-TNP (**Fig 1**) or Nase-TNP (not shown) failed to sensitize for CHS.

The observed differences in the ability of peptides to transfer CHS responses reflect the differential loading of peptide on to surface MHC molecules In order to determine whether the differences in the ability of hapten-modified peptide-loaded DC to transfer CHS responses *in vivo* could be attributed to the avidity of peptides for MHC molecules on the cell surface, DC were incubated with the various peptides and then the TNP expression on the cells was determined by fluorescence-activated cell sorter (**Fig 2**). TNP was clearly detectable, using a TNP-specific antiserum, on DC that had been incubated with the class I-binding peptides 04-TNP or 07-TNP (**Fig 2*A*, *B***). In contrast, TNP was not or barely detectable on cells incubated with the class II-binding peptide IgGVH-TNP (**Fig 2*C***).

DC incubated with class II-binding peptides have functional levels of TNP-modified peptides on their cell surface The data in **Fig 2** suggested that I-A^b specific peptides were failing to bind, or were binding poorly to MHC class II molecules on DC. Hence, it was of interest to determine whether class II-binding peptides were functionally present on these cells. We used the $CD8^+$ T cell hybridoma IA-8, which was H2-K^b restricted and specific for TNP in position 4 (von Bonin *et al*, 1993), and hybridoma IT/H6-A11, which was $CD4^+$ and specific for IgGVH-TNP in the context of I-A^b (Kohler *et al*, 1997). As shown in **Fig 3**, both hybridomas secreted IL-2 in response to TNBS-modified DC. In addition, I-A8 responded to DC modified with 04-TNP, but not with IgGVH-TNP, whereas IT/H6-A11 responded to DC pulsed with IgGVH-TNP but not with 04-TNP (**Fig 3**).

Sensitization with TNP-peptide/IFA or TNBS-DC results in preferential priming of $CD8^+$ T cells To identify the effector cells induced after s.c. injection of peptide/IFA emulsions or intradermal injection of TNP-peptide modified DC, we analyzed axillary lymph node cells 5 d after immunization. The identification of the effector cells induced by these protocols proved difficult when a complete contact hypersensitivity experiment was performed. As shown in **Fig 4**, a single epicutaneous TNCB application on the ears of control mice injected with unmodified DC efficiently primed TNP-specific cytolytic T cells in the draining lymph nodes, but not in spleen. Therefore, we decided to analyze the cells prior to ear challenge as this regimen allows the identification of T cells induced by the peptide or DC injections. The data in **Fig 5** show IFN-γ production by axillary lymph node cells. After s.c. injection of TNP peptide/IFA emulsions we found that the H-2K^b-binding peptide 04-TNP specifically induced IFN-γ producing $CD8^+$ T cells. Surprisingly, the I-A^b-binding peptide Nase-TNP also induced IFN-γ production by $CD8^+$ T cells but only very inefficiently by $CD4^+$ T cells (**Fig 5*A***). When mice were immunized with intradermal injection of 04-TNP-modified DC it was again found that $CD8^+$ T cells and no $CD4^+$ T cells were induced. Nase-TNP-DC immunization also resulted in the induction of $CD8^+$ T cells. In the CD4 population we detected nonspecific IFN-γ production, which was very high upon 04-TNP-DC immunization (**Fig 5*B***).

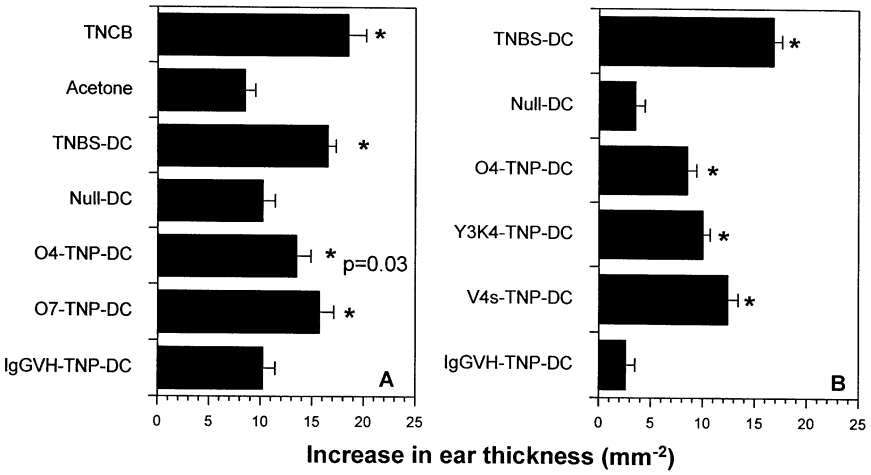

Figure 1. Hapten-modified MHC class I binding peptides transfer CHS *in vivo* when presented on DC. Groups of mice (n=3) were painted on the abdomen with 100 µl 7% TNCB or with the same volume of vehicle (acetone). The abdomens of the remaining groups were shaved. DC were derivatized with TNBS or pulsed with the indicated TNP peptides. On 2 consecutive days 3×10^5 DC were injected intracutaneously into the abdomen (*A*) whereas in (*B*), DC were injected once only. On day 5 after sensitization the ears of all mice were measured and then challenged with 20 µl 1% TNCB, 24, 48, and 72 h later the ears were re-measured, and the increase in each individual ear thickness was calculated. Data show the mean increase in ear thickness ±SEM n=6 ears for the 24 h time point. Differences in the mean values among treatment groups were determined using an unpaired Student's t test in Sigmastat 1.0 for PC (SPSS Software, Munich, Germany). Groups that showed significant difference in ear swelling from the negative control (acetone in *A*, Null-DC in *B*) were marked with an asterisk, $p<0.01$ unless otherwise stated.

DISCUSSION

Previous attempts to determine the importance of T cell subsets in CHS responses provide conflicting data on the role of $CD4^+$ and $CD8^+$ T cells. There is no doubt that $CD8^+$ T cells are effective mediators of CHS responses (Gocinski and Tigelaar, 1990; Bour *et al*, 1995; Xu *et al*, 1996, 1997; Kolesaric *et al*, 1997), and can develop without the need for $CD4^+$ T cell help (Xu *et al*, 1997; Kolesaric *et al*, 1997). There is, however, conflicting evidence for the role of $CD4^+$ T cells in CHS. With some studies suggesting a purely regulatory role for this subset (Bour *et al*, 1995; Xu *et al*, 1996, 1997; Kolesaric *et al*, 1997) and others (Hauser, 1990; Gocinski and Tigelaar, 1990; Kohler *et al*, 1995; Kondo *et al*, 1996) implicating $CD4^+$ T cells as an effector population. Hence, we were interested in using a novel system to approach this question. The initial experiments, in which peptides were injected s.c. as an emulsion in IFA, supported a role for $CD4^+$ T cell mediated immunity in CHS, with the induction of low but specific CHS responses following the injection of the MHC class II binding peptides Nase-TNP, PCC-TNP, IgGVH-TNP, or CH-7-TNP (**Table I**) (Kohler *et al*, 1995; Weltzien *et al*, 1996), but not by the MHC class I-binding peptides Y3K4-TNP or V4s-TNP (**Table I**). We observed, however, contrasting results when BM-DC were pulsed with peptide and injected into syngeneic recipients. In this case, DC pulsed with class I-binding haptenized peptides sensitized for CHS responses as effectively as epicutaneous hapten painting (**Fig 1**), implicating $CD8^+$ T cells as potent effector cells in CHS, a result which is in agreement with previous studies (Gocinski and Tigelaar, 1990; Bour *et al*, 1995; Xu *et al*, 1996, 1997; Kolesaric *et al*, 1997). In contrast, DC pulsed with the MHC class II-binding peptides IgGVH-TNP or Nase-TNP (not shown), usually failed to induce CHS. Occasionally, however, injection of IgGVH-TNP pulsed DC also resulted in ear swelling responses (data not shown), indicating a borderline reaction. Thus, depending on the mode of application, both MHC class I-specific as well as class II-specific TNP peptides may sensitize for CHS responses *in vivo*.

The finding that CHS sensitization with MHC class I-specific and class II-specific, TNP-derivatized peptides was dependent on the mode of application, i.e., s.c. injection in emulsion with IFA versus pulsed DC, raises interesting questions. One possible explanation for the failure of DC pulsed with MHC class II-binding peptides to sensitize for CHS, is that MHC class II loading by the external addition of peptides is significantly less efficient than MHC class I loading. This view is substantiated by fluorescent staining of haptenized cells with TNP-specific anti-serum, which failed to detect TNP on DC following incubation with the TNP-modified MHC class II-binding peptide IgGVH-TNP (**Fig 2**). In fact, we never succeeded in achieving significant staining of any MHC class II binding TNP peptide on APC, whether fixed or unfixed (data not shown). Thus the numbers of MHC class II associated TNP determinants on DC's pulsed with soluble TNP peptide, may be at the limits required for the activation of naive $CD4^+$ T cells explaining the rare positive results. Alternatively, the low levels of TNP on the cell surface of IgGVH-TNP-pulsed DC, could reflect the high turnover of MHC class II in these cells (Cella *et al*, 1997). Functional data comparing fixed and unfixed APC in T cell stimulation assays, however, showed no significant difference between these two APC populations (Weltzien *et al*, 1996) suggesting that a high turnover of TNP peptide/MHC class II complexes does not account for the lack of staining as seen in **Fig 2**. Certainly, the ability of IgGVH-TNP-pulsed DC to stimulate IL-2 production from the hybridoma IT/H6-A11 suggests that the peptide is present at functional levels on the cell surface (**Fig 3**).

In contrast, the oil emulsions of peptide in IFA may reduce the amounts of free peptide available to load MHC class I molecules on the surface of Langerhans cells in the skin. The emulsion of TNP peptide is probably internalized as microdroplets by endocytosis, favoring the loading of class II rather than class I molecules. This may explain why class I MHC-binding TNP peptides were ineffective in sensitizing for CHS when administered as an emulsion in IFA. On the other hand, the ability of class II-binding peptides to transfer CHS following application in IFA but not in the context of DC, may reflect the "physiologic" loading of MHC class II within the endosomal compartment of Langerhans cells. This loading may be quantitatively and, more importantly, functionally more efficient than external loading by soluble peptides. A second possibility is that class II-binding peptides, but not class I-binding peptides, participate in delivering a signal causing the Langerhans cells to migrate to the draining lymph node. Certainly in macrophages, the ligation of MHC class II, but not MHC class I, results in intracellular calcium flux (Dialynas *et al*, 1997). Arguing against the idea that MHC class II ligation is required for DC migration, is the fact that injection of DC pulsed with MHC class I-binding peptides induced CHS responses. This may, however, reflect differences in the requirement for migration signals between *in situ* Langerhans cells and injected DC.

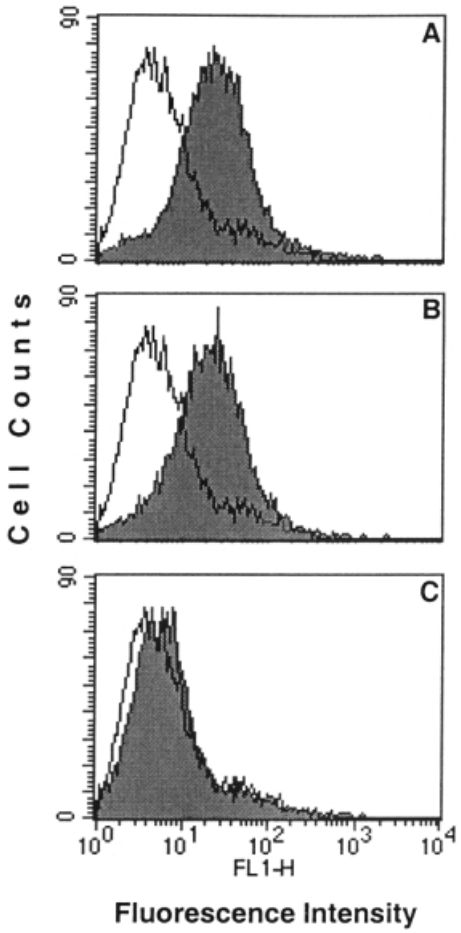

Figure 2. Hapten-modified MHC class I-binding peptides, but not the class II-binding peptides, are detectable on peptide pulsed DC. DC were incubated for 2 h at 37°C with either medium alone or 100 μg 04-TNP per ml (*A*), 100 μg 07-TNP per ml (*B*), or 100 μg IgGVH-TNP per ml (*C*). The DC were washed thoroughly, and incubated with rabbit anti-TNP serum followed by FITC second antibody, for details see *Materials and Methods*. A gate was placed around the phycoerythrin-labeled $CD11c^+$ population and the FITC fluorescence was analyzed by FACScan. Open histograms show staining in the absence, filled histograms in the presence of peptides.

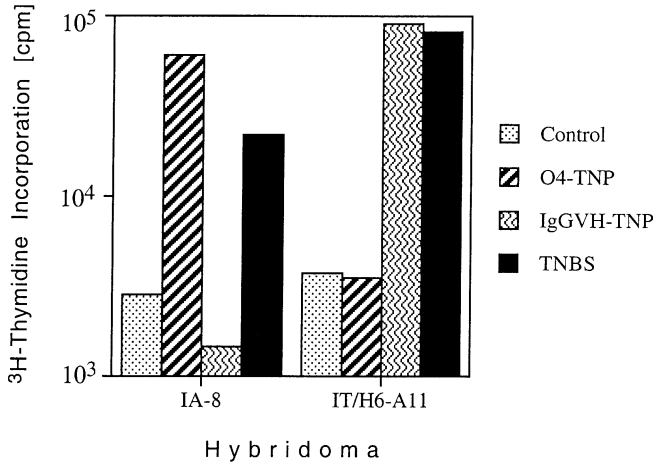

Figure 3. Antigen-specific IL-2 induction by peptide-pulsed DC. Gamma-irradiated (3×10^3 rad) DC were incubated for 2 h at 37°C with cRPMI (control) or 100 μM of peptide (04-TNP or IgGVH-TNP). TNBS-DC were derivatized by incubation with 1 mM TNBS for 10 min. DC were used as stimulator cells for the hybridomas I-A8 (TNP in position 4, K^b-restricted) and IT/H6-A11 (IgGVH-TNP, I-A^b-restricted). Supernatants were collected after 24 h stimulation and IL-2 production was quantified using IL-2 dependent cell line (CTLL). Data represent means of triplicates with SD <12%.

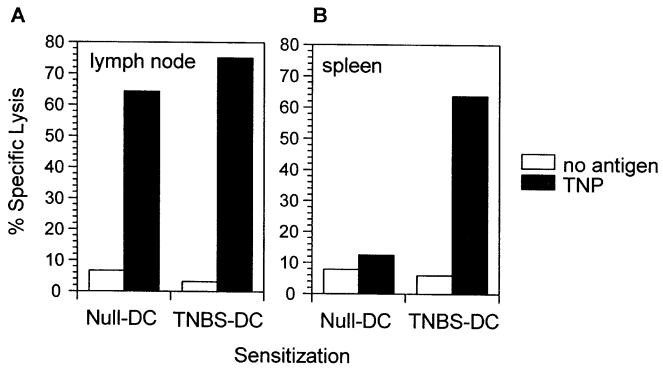

Figure 4. Induction of cytotoxic TNP-specific $CD8^+$ T cells in DC-TNP induced CHS. Mice were immunized with untreated or TNBS-modified DC. After 5 d, ears were challenged with 1% TNCB acetone. Seventy-two hours later, spleen and lymph nodes were removed and restimulated with TNBS-modified syngeneic spleen cells for 3d . EL4 target cells were labeled with ^{51}chromium for 90 min and modified with TNBS or left untreated. Pooled auricular, maxillary, and axillary lymph node (*A*) or spleen cells (*B*) were added to the target cells at an effector/target ratio (E/T) of 30:1 and a standard 4 h chromium release assay performed.

In order to directly analyze the effector cells induced by the different immunization protocols we analyzed the cytokine production of lymph node cells after immunization prior to ear challenge. This avoids the complication that effector cells are already efficiently induced by ear challenge with TNCB as shown in **Fig 4**. We additionally analyzed the effector population after CHS induction and obtained similar results (data not shown). Our experiments clearly showed that s.c. injection of the class I binding peptide 04-TNP in IFA induced antigen-specific $CD8^+$ T cells which produce IFN-γ in response to TNP. Similarly, intradermal injection of the BM-DC loaded with this peptide induced $CD8^+$ T cells. In contrast when the MHC class II binding peptide Nase-TNP in IFA or Nase-TNP-DC were injected we found an unexpected result. $CD8^+$ T cells were induced in both cases. For CD4 T cells a slight increase of IFN-γ production was seen only after peptide/IFA injection. No significant IL-4 production was detectable after peptide/IFA injection. After DC immunization, however, background IL-4 production (1 ng per ml) was seen after Nase-TNP-DC immunization and to a lesser extent after control DC injection (600 pg per ml) (data not shown).

The mechanism of priming $CD8^+$ T cells by Nase-TNP is at present unclear. A direct binding of this peptide to MHC class I molecules has not been demonstrated but cannot be ruled out, although the lack of staining with TNP-specific antibodies (**Fig 2**) would suggest a low-affinity binding. In the case of peptide/IFA emulsions it is possible that some of the peptide reaches the cytosol of an antigen-presenting cell due to the high local concentration or is taken up like liposome-encapsulated peptides and then trimmed to fit into the binding groove of MHC class I molecules. In fact, the nonamer of positions 3–11 in the Nase peptide contains a potential binding motif for H-2D^b ($V_5N_6L_9$) (Falk *et al*, 1990). We have previously shown that MHC class I binding peptides can be generated from a TNP-modified protein, delivered to the cytosol via liposomes and that such TNP peptides are efficiently loaded on to MHC class I molecules and reach the cell surface (Martin *et al*, 1993b). A further possibility is a leakiness of the MHC class II antigen processing and presentation pathway allowing for a delivery of endocytosed material to the cytosol. This could explain why Nase-TNP pulsed DC can prime a CD8 T cell response.

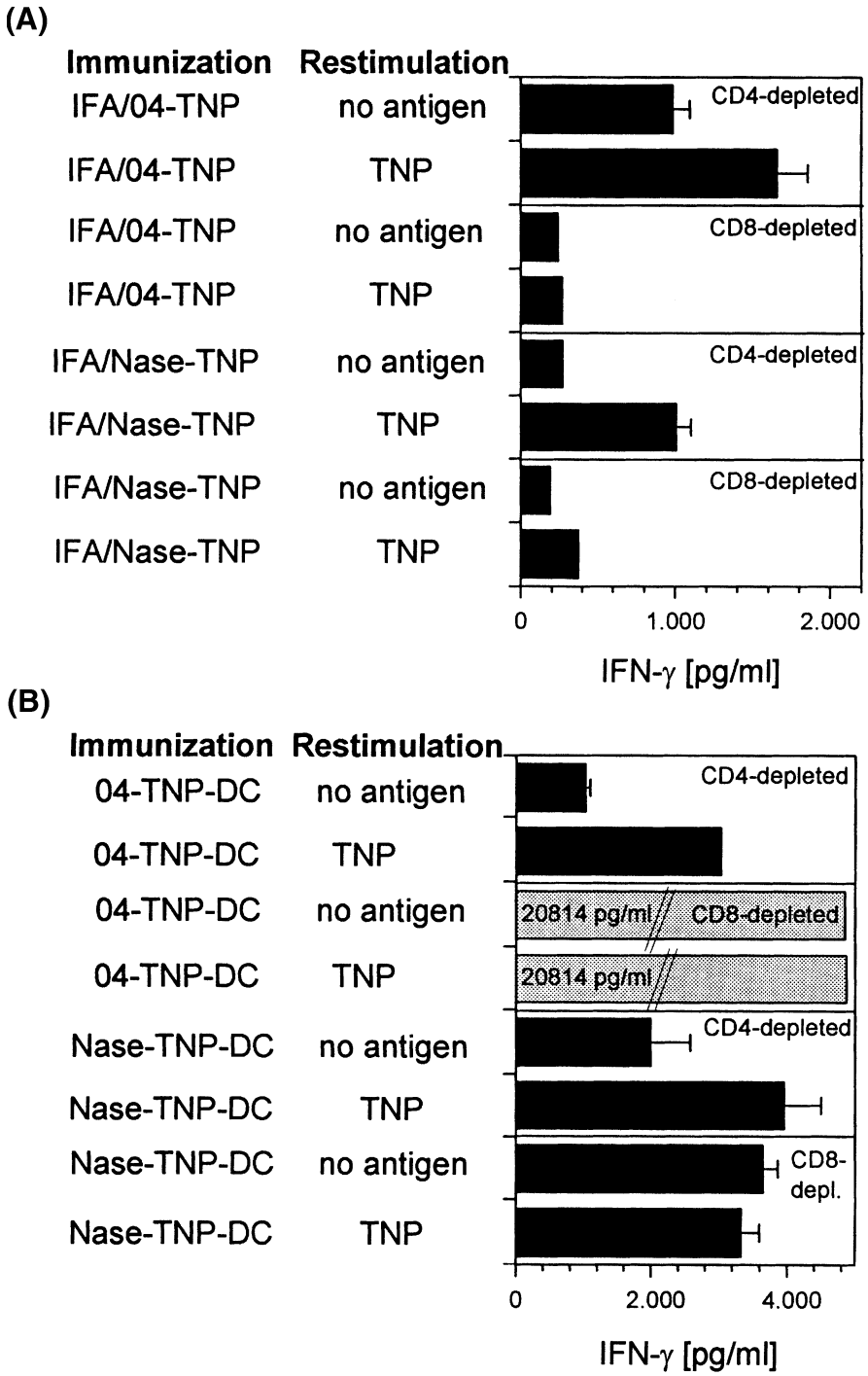

Figure 5. Analysis of the effector T cells in TNP-mediated CHS induced by different immunization protocols. (*A*) Mice were sensitized by s.c. injection of peptide/IFA emulsions. Five days later axillary lymph nodes were removed and CD4 or CD8 T cells depleted. Cells were restimulated *in vitro* for 36 h with syngeneic untreated (no antigen) or TNP-modified spleen cells. Culture supernatants were harvested and an IFN-γ ELISA performed. (*B*) Mice were sensitized by intradermal injection of TNP-peptide modified DC. Five days later axillary lymph nodes were removed and the experiment performed as in (*A*).

There is no evidence that the covalently bound TNP moiety can be cleaved from the carrier peptide and be transferred to other, e.g., MHC class I-binding peptides.

Interestingly, despite the activation of $CD8^+$ T cells by Nase-TNP in IFA or Nase-TNP-DC we only see an ear swelling response in the case of the peptide/IFA emulsion (**Fig 1**). This may be explained by a contribution of antigen-specific $CD4^+$ T cells of the Th1 type, which are probably not induced by Nase-TNP-DC immunization in the sensitization phase (**Fig 5**) or background IL-4 production after DC immunization seen in the CD8-depleted population (data not shown). In the case of DC injections we also find a strongly enhanced background level of IFN-γ production in the CD8-depleted population. These findings demonstrate that the IFN-γ production alone is not sufficient to trigger a CHS response.

Thus it appears that the mode of peptide application, and hence the mechanism of MHC loading, determines whether $CD8^+$ or $CD4^+$ T cells dominate a CHS reaction. Certainly under the right conditions, MHC class II-binding peptides were able to sensitize low TNP-specific CHS responses in unmanipulated mice. In the context of peptide presentation by injection of DC, however, only MHC class I-binding peptides but not MHC class II-binding peptides reproducibly induced strong CHS responses. In this context, it was particularly surprising that the two K^b-binding peptides 04-TNP and 07-TNP induced comparable CHS responses. From *in vitro* T cell cloning experiments we had previously shown that TNP in position 4 of the K^b-binding octapeptides (represented by 04-TNP) comprised a major and dominant type of TNP epitope on TNBS-modified cells (Martin

et al, 1992, 1993). Moreover, we showed that T cell recognition of the TNP epitope in position 4, was quite independent of the amino acid sequence of the carrier peptide, whereas for TNP epitopes in position 7 (represented by 07-TNP) recognition was highly dependent on the specificity of the carrier sequence (Martin *et al*, 1993a). We therefore hypothesized that TNP determinants positioned on the central amino acid 4 in K^b-associated peptides represented a dominant determinant in CHS induction (Martin *et al*, 1993). This is clearly at odds with the data shown in **Fig 1**, which suggest that 07-TNP was at least as effective as 04-TNP in the induction of CHS. As one possible explanation for this discrepancy we recently found (Preckel *et al*, 1998) that carrier cross-reactivities for position 4-modified TNP peptides were limited to the cytotoxic but not to the proliferative responses of the corresponding T cell clones. Thus in terms of cell proliferation, T cells specific for TNP in position 4 were no more cross-reactive than those specific for position 7. As one of the important functions of antigen-loaded DC, upon entry into a draining lymph node, is to induce the proliferation of reactive T cell clones, the antigen restriction of T cell proliferation may explain the comparable efficiency of CHS transfer by 04-TNP and 07-TNP.

In conclusion, we have demonstrated that in mice with unmanipulated immune systems both MHC class I-binding and II-binding TNP peptides can induce effector T cells for CHS responses with an unexpected $CD8^+$ T cell response when MHC class II-binding peptides were used. Following skin painting with hapten, self peptides in both MHC class I and II grooves will be derivatized. Our data, in agreement with others (Gocinski and Tigelaar, 1990; Bour *et al*, 1995; Xu *et al*, 1996, 1997; Kolesaric *et al*, 1997) suggest that the major effector signal, given by derivatized DC, will be through the MHC class I pathway even when class II epitopes are used. In contrast, following s.c. injection of peptide in IFA, MHC class II but not class I-binding peptides induce CHS responses. In this case, $CD8^+$ T cells were primed by the class II-binding peptides and may exert the effector functions necessary for CHS induction. We also observed a weak priming of $CD4^+$ T cells by this protocol that may support the CD8 response. This suggests, in agreement with studies in $CD4^+$ T lymphocyte deficient mice (Kondo *et al*, 1996), and previous studies with MHC class II-binding peptides (Kohler *et al*, 1995; Weltzien *et al*, 1996), that under certain circumstances $CD4^+$ T cells can act as effector cells in CHS responses but the allergic response is clearly dominated by $CD8^+$ T cells.

This work was supported by the Klinische Forschergruppe ''Pathomechanismen der allergischen Entzündung'' BMBF FKZ: 01GC9701/7 and Deutsche Forschungsgemeinschaft, SFB 388.

REFERENCES

von Bonin A, Ortmann B, Martin S, Weltzien HU: Peptide conjugated hapten groups are the major antigenic determinants for trinitrophenyl-specific cytotoxic T cells. *Int Immunol* 4:869–874, 1992

von Bonin A, Martin S, Plaga S, Hebbelmann S, Weltzien HU: Purified MHC class I molecules present hapten-conjugated peptides to TNP/H-$2K^b$-specific T-cell hybridomas. *Immunol Lett* 35:63–68, 1993

Bour H, Peyron E, Gaucherand M, *et al*: Major histocompatibility complex class I-restricted $CD8^+$ and class II-restricted $CD4^+$ T cells, respectively, mediate and regulate contact sensitivity to dinitrofluorobenzene. *Eur J Immunol* 25: 3006–3010, 1995

Cavani A, Hackett CJ, Wilson KJ, Rothbard JB, Katz SI: Characterization of epitopes recognized by hapten-specific $CD4^+$ T cells. *J Immunol* 154:1232–1238, 1995

Cella M, Engering A, Pinet V, Pieters J, Lanzavecchia A: Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* 388:782–787, 1997

Dialynas DP, Tan PC, Yu J: Cytokine modulatable signaling through macrophage HLA class II. 1. IFN-gamma upregulates the efficiency of Ca^{2+} mobilization in response to ligation of macrophage HLA-DP. *J Interferon Cytokine Res* 17: 671–679, 1997

Falk K, Rötzschke O, Stefanovic S, Jung G, Rammensee HG: Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 351:290–296, 1990

Gocinski BL, Tigelaar RE: Roles of $CD4^+$ and $CD8^+$ T cells in murine contact sensitivity revealed by in vivo monoclonal antibody depletion. *J Immunol* 144:4121–4128, 1990

Grabstein K, Eisenman J, Mochizuki D, *et al*: Purification to homogeneity of B cell stimulatory factor. A molecule that stimulates proliferation of multiple lymphokine-dependent cell proliferation. *J Exp Med* 163:1405–1414, 1986

Hauser C: Cultured epidermal Langerhans cells activate effector T cells for contact sensitivity. *J Invest Dermatol* 4:436–440, 1990

Inaba K, Inaba M, Romani N, *et al*: Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 176:1693–1702, 1992

Kohler J, Martin S, Pflugfelder U, Ruh H, Vollmer J, Weltzien HU: Cross-reactive trinitrophenylated peptides as antigens for class-II major histocompatibility complex-restricted T-cells and inducers of contact hypersensitivity in mice—limited T-cell receptor repertoire. *Eur J Immunol* 25:92–101, 1995

Kohler J, Hartmann U, Grimm R, Pflugfelder U, Weltzien HU: Carrier-independent hapten recognition and promiscuous MHC restriction by CD4 T cells induced by trinitrophenylated peptides. *J Immunol* 158:591–597, 1997

Kolesaric A, Stingl G, Elbe-Burger A: MHC class I^+/II^- dendritic cells induce hapten-specific immune responses *in vitro* and *in vivo*. *J Invest Dermatol* 109: 580–585, 1997

Kondo S, Beissert S, Wang B, *et al*: Hyporesponsiveness in contact hypersensitivity and irritant contact dermatitis in CD4 gene targeted mouse. *J Invest Dermatol* 106:993–1000, 1996

Martin S, Ortmann B, Pflugfelder U, Birsner U, Weltzien HU: Role of hapten-anchoring peptides in defining hapten-epitopes for MHC-restricted cytotoxic T cells. Cross reactive TNP-determinants on different peptides. *J Immunol* 149:2569–2575, 1992

Martin S, von Bonin A, Fessler C, Pflugfelder U, Weltzien HU: Structural complexity of antigenic determinants for class I MHC-restricted hapten-specific T cells: two qualitatively differing types of H-$2K^b$-restricted TNP epitopes. *J Immunol* 151:678–687, 1993a

Martin S, Niedermann G, Leipner C, Eichmann K, Weltzien HU: Intracellular processing of hapten-modified protein for MHC class I presentation: cytoplasmic delivery by pH-sensitive liposomes. *Immunol Lett* 37:97–102, 1993b

Martin S, Ruh H, Hebbelmann S, Pflugfelder U, Rüde B, Weltzien HU: Carrier-reactive hapten-specific cytotoxic T lymphocyte clones originate from a highly preselected T cell repertoire: implications for chemical-induced self-reactivity. *Eur J Immunol* 25:2788–2796, 1995

Preckel T, Grimm R, Martin S, Weltzien HU: Altered hapten ligands antagonize trinitrophenyl-specific cytotoxic T cells and block internalization of hapten-specific receptors. *J Exp Med* 185:1803–1813, 1997

Preckel T, Breloer M, Kohler H, von Bonin A, Weltzien HU: Partial agonism and independent modulation of T cell receptor and CD8 in hapten-specific cytotoxic T cells. *Eur J Immunol* 28:3706–3718, 1998

Weltzien HU, Moulon C, Martin S, Padovan E, Hartmann U, Kohler J: T-cell immune responses to haptens—Structural models for allergic and autoimmune reactions. *Toxicology* 107:141–151, 1996

Xu H, Dilulio NA, Fairchild RL: T cell populations primed by hapten sensitization in contact hypersensitivity are distinguished by polarized patterns of cytokine production-interferon gamma producing (Tc1) effector $CD8^+$ T-cells and interleukin (IL)-4/IL-10-producing Th2 negative regulatory $CD4^+$ T-cells. *J Exp Med* 183:1001–1012, 1996

Xu H, Banergee A, Dilulio NA, Fairchild RL: Development of effector $CD8^+$ T cells in contact hypersensitivity occurs independently of $CD4^+$ T cells. *J Immunol* 158:4721–4728, 1997